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The translational mobility of substances within the cytoplasmic matrix

(cytoplasm/cytoskeleton/photobleaching/fluorescent probes)

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ABSTRACT **The translational mobility of fluorescent-labeled molecules injected into the cytoplasm of living cells can be measured by the fluorescence recovery after photobleaching (FRAP) technique. In the fibroblast cytoplasm, the diffusion coefficients, *D*, of test macromolecules ranging in molecular weight from 12,000 to 440,000 are about 10^{-8} cm^2/sec and exhibit almost no dependence on molecular weight. FRAP experiments also showed that macromolecular diffusion within Sepharose beads having an effective pore size smaller than the "microtrabecular lattice" is only slightly retarded compared to buffer values—in contrast to the marked retardation measured in the cytoplasm. This leads to the conclusion that diffusion in the cytomatrix is dominated not by steric effects but rather by binding of the diffusing species to elements of the cytomatrix. These diffusion rates were difficult to modulate; cytochalasin, colchicine (except at 5°C), and taxol treatments had little effect. The diffusion rates were not dependent on cellular energy metabolism. However, hypotonic treatment increased the *D* for bovine serum albumin by nearly 2-fold, whereas hypertonic treatment halved *D*. Withdrawing the free water from the cell by using 44% polyethylene glycol treatment stopped the translational mobility of the test molecules. A survey of the recent literature is presented, which shows that major differences in the cytomatrix of different cell types exist with respect to the translational diffusion of injected probes. Finally, the spectrum of cytoplasmic translational mobilities ranging from small molecules to organelles is discussed.**

The structure of the cytomatrix is a subject of renewed interest due, in part, to advances in electron microscopic (EM) technology. Current views range from a more classical fibrous meshwork composed only of the three major filament systems (actin fibers, microtubules, and intermediate filaments) and their associated proteins (1, 2) to a dense network embracing, at least transiently, almost all of the proteins of the cytoplasm and termed the "microtrabecular lattice" (MTL) (3–5). Furthermore, at least for the purposes of this work, the large collection of intracellular membranes must be considered an integral part of the cytomatrix. The formation of portions of the filamentous network could be driven by the generally high cytoplasmic protein concentration (6), and elements of this network may serve to coordinate some biochemical reactions (7). The conflicting structural views do have a common thread: they depict a cytoplasm organized with a surprising degree of intricacy.

Novel methods to study the diffusion and distribution of molecules within large, living cells have been developed with the rationale that studies of the motion of macromolecules in the cytoplasm will provide information concerning the structure and apparent viscosity of this compartment (8–11). Recently, the photobleaching techniques have been used to measure the diffusion of exogenously injected foreign and native macromolecules within the cytoplasm of living cells (12–15). The apparent diffusion coefficient, *D*, of fluorescein-conjugated bovine serum albumin and nonspecific fluorescein-conjugated IgG has been measured in fibroblasts to be approximately 10^{-8} cm^2/sec at 25°C and was found to undergo a 5-fold increase in the range of 5–37°C (12). In this study, we examine the modulation of cytoplasmic *D*s produced by changes in tonicity, diffusant size, and cytoskeletal structure.

MATERIALS AND METHODS

Reagents. The fluorescent probe 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF) was purchased from Research Organics (Cleveland, OH) and was conjugated to apoferritin, insulin, and bovine serum albumin, all of which were purchased from Sigma. Colchicine, cytochalasin D, 2-deoxyglucose, and Triton X-100 also were purchased from Sigma. Sodium azide was obtained from Fisher, Gelvatol (polyvinyl alcohol), from Monsanto, goat anti-rat IgG and $F(ab')_2$ anti-rat fragments, from Cappel Laboratories (Cochranville, PA), NBD-phallacidin (7-nitrobenz-2-oxa-1,3-diazole-conjugated phallacidin), from Molecular Probes (Junction City, OR), and Sepharose CL-4B beads, from Pharmacia. The anti-tubulin antibody, nocodazole, and taxol were all gifts of Brian Herman (University of North Carolina at Chapel Hill). Fluorescein isothiocyanate (FITC)-conjugated-goat anti-rabbit IgG was a gift of Keith Burridge (University of North Carolina at Chapel Hill).

Cells, Microinjection, and Treatments. BG-9 human diploid fibroblast cells were cultured and microinjected *en masse* by the erythrocyte-mediated microinjection technique as described (12) or singly by needle microinjection, essentially following the Graessmann technique (16) using Leitz micromanipulators and an inverted microscope generously provided by K. Burridge. After microinjection, cells were incubated at 37°C for 15 min prior to fluorescence recovery after photobleaching (FRAP) or further treatments. The details of the various treatments applied to the cells are given in the figure captions and footnotes to the tables.

Immunofluorescence and Photobleaching. Cells to be labeled with anti-tubulin to visualize the microtubule network were rinsed briefly with phosphate-buffered saline (P_i/NaCl) and fixed in 3.7% formaldehyde in P_i/NaCl for 10 min at room temperature. After a rinse with P_i/NaCl, the cells were further fixed and rendered permeable by immersion in ace-

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Abbreviations: EM, electron microscopy/electron microscopic; FRAP, fluorescence recovery after photobleaching; MTL, microtrabecular lattice; FITC, fluorescein isothiocyanate; FITC-IgG, FITC-conjugated IgG; DTAF, 5-(4,6-dichlorotriazin-2-yl)aminofluorescein; DTAF-albumin, DTAF-conjugated bovine serum albumin; NBD, nitrobenz-2-oxa-1,3-diazole.

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tone for 2 min at −20°C. After an extensive wash in P_i/NaCl, the cells were labeled with a 1:50 dilution of the rabbit anti-tubulin serum for 30 min at 37°C, washed extensively in P_i/NaCl, and incubated with FITC-conjugated goat anti-rabbit IgG (FITC-IgG) for 30 min at 37°C. Cells to be labeled with NBD-phallacidin to visualize the actin network were rinsed briefly in P_i/NaCl and fixed in 3.7% formaldehyde in P_i/NaCl for 30 min at room temperature. After an extensive rinse in P_i/NaCl, the cells were permeabilized by a 5-min incubation in 0.1% Triton X-100 in P_i/NaCl. After an extensive wash in P_i/NaCl, cells were incubated with 8.25 ng of NBD-phallacidin per coverslip for 30 min at 37°C. After the labeling procedures, all cells were washed in P_i/NaCl, rinsed in distilled H_2O, and mounted on slides with Gelvatol.

The FRAP setup as applied to cytoplasmic measurements has been described in detail (17, 18).

RESULTS AND DISCUSSION

Photodamage Artifacts. The use of the photobleaching techniques has raised the question of photodamage artifacts, particularly in the photobleaching step (19, 20). Several arguments suggest that the diffusion rates quoted in this article are largely free from such artifacts. First, partially prebleaching the fluorescence over the entire cell by using a defocused laser beam does not change diffusion values obtained from subsequent spot photobleaching measurements. Second, the values obtained by FRAP are consistent with the rates of fluorescein-conjugated albumin spreading from the site of erythrocyte-mediated microinjection (12), which is monitored without photobleaching.

Modulation of the Diffusion Rates of Exogenous Macromolecules in the Cytoplasm. *Size and nature of the diffusant.* Cytoplasmic *D*s for a variety of diffusing species within the fibroblast cytoplasm are reported in Table 1. Apart from the small molecule carboxyfluorescein, all other macromolecules diffuse in the range of 1–2 × 10^{-8} cm^2/sec. In fact, the smallest diffuser, insulin, appears to be the slowest diffuser. Insulin was chosen because it has been shown not to interact with F-actin (21), yet judging from the low value of its *D*, it appeared to interact with the fibroblast cytomatrix. To counter the argument that an IgG (by virtue of the F_c fragment portion) is intrinsically adherent, we tested the $F(ab')_2$ fragment. These fragments did not diffuse appreciably faster. Even the larger apoferritin diffused at a rate similar to those of the smaller macromolecules. These data clearly do not support a model of the cytoplasm as a simple viscous solution in which diffusion is described by the Stokes–Einstein equation; for such a model, we would expect the *D*s to depend inversely on molecular radius. It does suggest a similar diffusion rate limitation for all of the macromolecular species tested.

Table 1. Measured cytoplasmic *D* as a function of the size and nature of the diffusing species

Diffusing species	M_r	Radius,* Å	Cytoplasmic *D* (± SEM), cm^2/sec	Mobile fraction, % ± SEM
Carboxyfluorescein†	400	≈6.5	>4 × 10^{-8}	—
Insulin	12,000	≈16.0	0.9 (±0.07) × 10^{-8}	87 ± 3
Bovine serum albumin	67,000	35.5	1.7 (±0.09) × 10^{-8}	97 ± 1
$F(ab')_2$	100,000	40.6	1.6 (±0.13) × 10^{-8}	95 ± 2
Apoferritin	440,000	61.0	1.6 (±0.14) × 10^{-8}	96 ± 2

*The molecular radii were estimated from that of bovine serum albumin and assumed that radius is proportional to (molecular weight)$^{1/3}$. Bovine serum albumin and ferritin radii were taken from the Sephadex manual.

†As previously reported (12).

Structure of the medium. Fig. 1 shows a comparison of the diffusion rates of $F(ab')_2$ fragments within Sepharose 2B-CL beads (as a model for an inert cytomatrix structure) and in the actual fibroblast cytoplasm [in reference to the rapid diffusion occurring through aqueous buffer ($D \approx 6 \times 10^{-7}$ cm^2/sec)]. Diffusion of $F(ab')_2$ fragments through Sepharose 2B-CL beads having a mean pore diameter of ≈500 Å is reduced to ≈1/2, but not to nearly the extent observed in the human fibroblast cytoplasm. The estimated effective pore size of the MTL in PTK and NRK cultured cells is in the range of 720–980 Å (23) and is actually greater than that of the Sepharose beads. Thus, this experiment supports the assertion of Gershon *et al.* (23) that the volume fraction occupied by the cytoplasmic matrix is too small to impede diffusion to the extent observed and leads to the hypothesis that the probes must bind transiently to the elements of the cytomatrix to explain their slow diffusion rates.

Tonicity variation. The tonicity of the medium bathing the cells had an effect on the cytoplasmic diffusion of DTAF-conjugated bovine serum albumin (DTAF-albumin) (Fig. 2). Control *D*s were measured when the cells were bathed in isotonic sodium chloride. When the cells were placed into hypertonic salt solution (1.2 osM), the *D* for DTAF-albumin was decreased to 60% of its control value. Furthermore, DTAF-albumin became operationally immobile after treatment of the cells with 44% polyethylene glycol to provide extreme hypertonicity (24). This effect was reversible, as washing the polyethylene glycol from the cultures allowed the DTAF-albumin to become mobile again. These experiments suggested that free water in the cytoplasm was required for cytoplasmic diffusion to occur. On the other hand, hypotonic treatment (0.17 osM) increased the value of *D* to 170% of control.

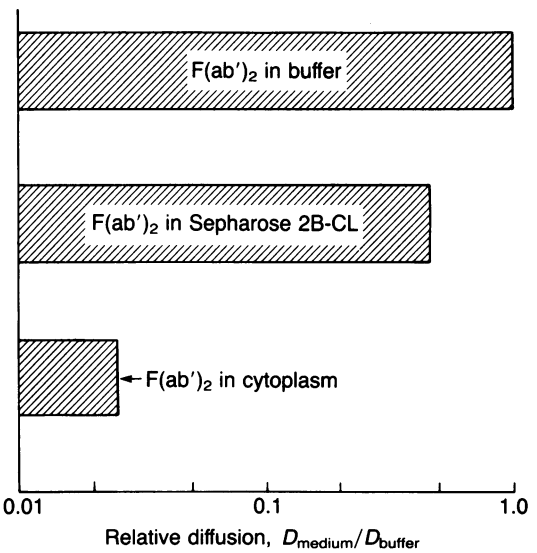

FIG. 1. Relationship of the diffusion rates of DTAF-conjugated $F(ab')_2$ in buffer (top bar), within Sepharose 2B-CL beads (middle bar), and in the cytoplasm of BG-9 cells (bottom bar). The buffer *D* was estimated from data of Barisas and Leuther (22). Measurement on beads was made by allowing beads to equilibrate with ≈1 μM DTAF-$F(ab')_2$ in microinjection buffer, gently squeezing beads and solution between a coverslip and slide, and photobleaching the largest beads (200-μm diameter) by using a beam radius, w_s, of 4.2 μm. Buffer regions adjacent to the beads were photobleached to give the aqueous diffusion values. (Absolute values of *D* are about a factor of 3 too low, presumably because of the thickness of the sample, which causes the beam to intercept parts of the sample having radii larger than the minimum radius; thus, the calculated *D* values are somewhat too low. Measurement is also sensitive to buffer and glass preparation, both of which can cause variable amounts of protein adsorption to the glass surfaces of the chamber.)

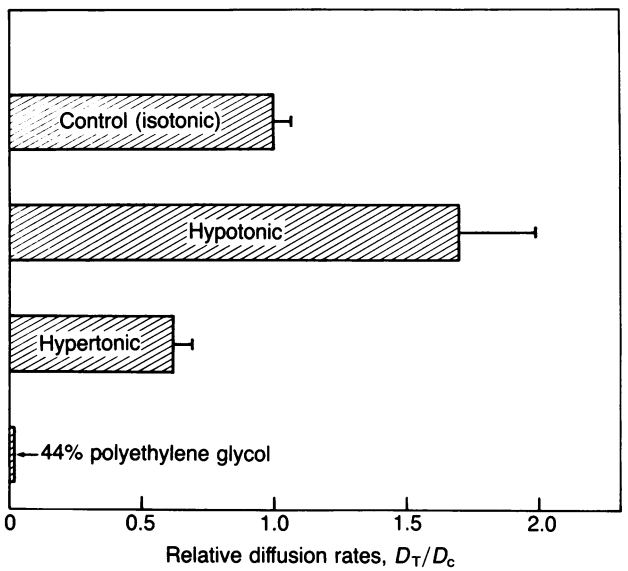

FIG. 2. Effect of tonicity on the cytoplasmic diffusion rates of DTAF-albumin [expressed as the ratio of the D in the treated sample (D_T) to the D in the isotonic control (D_c)]. Cells were loaded with DTAF-albumin by the erythrocyte-microinjection method (12) and then were exposed to hypotonic NaCl solution (0.17 osM) or hypertonic KCl (1.2 osM) for 15 min at room temperature prior to the FRAP measurement. Similar ratios were obtained when the experiment was repeated 18 months later using the needle microinjection technique to load the cells, although the control value (D_c) was increased from 1.0×10^{-8} cm^2/sec to 1.5×10^{-8} cm^2/sec. Incubation with 44% polyethylene glycol operationally immobilizes the probe as reported (24).

Chemical treatment. Treatment of cells with cytochalasin D to disrupt microfilaments failed to alter appreciably the cytoplasmic D for DTAF-albumin (Table 2, top row). The second row of Table 2 gives results of the previously reported experiment (12) showing that colchicine effects a 3-fold increase in D in cells previously cooled to 4°C. However, neither colchicine at room temperature (data not shown) nor taxol, which stabilizes microtubules (25), altered the cytoplasmic Ds of bovine serum albumin. Immunofluorescence studies indicated that these treatments, in fact, did alter the normal cytoskeletal arrangements for these cells (data not shown). Thus, at room temperature, major alterations of either microfilaments or microtubules do not alter appreciably the rate of cytoplasmic diffusion. Finally, cytoplasmic diffusion does not appear to depend on cellular energy sources (Table 2, bottom row) since nonmetabolizable glucose and inhibitors of oxidative phosphorylation have little effect on the measured diffusion rates.

Cytoplasmic structure. The cytoplasmic diffusion rates of test macromolecules appear relatively constant for a given cell type and are difficult to alter. However, a survey of the limited photobleaching literature reveals a large range of Ds of similar molecules in different cells (Table 3). Values for D of fluorescein-labeled bovine serum albumin range from 0.6×10^{-8} cm^2/sec in chicken gizzard cells (14) to 40×10^{-8} cm^2/sec in amoebae (13). This range of values indicates the ability of these macromolecular probes to report on the presumably large variations in cytoplasmic structure exhibited in these diverse cells.

Retarded diffusion of macromolecules within the cytoplasmic matrix. Recent studies have provided biochemical and ultrastructural evidence that strongly suggests that the view of the cytoplasm as a homogeneous, viscous medium is grossly inadequate. Rather, the current view of the cytoplasm is one of a matrix displaying considerable structural organization. Given this supposition, we can question how the organized cytoplasm restricts the apparent diffusion of injected macromolecules. Retardation by steric obstruction and/or binding to matrix and membraneous elements are the two obvious possibilities. Treatments of flow through porous media (26) tell us that the mean pore diameter must be of the order of the diameter of the diffusing species in order for the medium to have an appreciable effect on the rate of diffusion. With the assumption that the existence of the MTL is documented by high-voltage EM, calculations show that the intratrabecular space is of the order of 1000 Å (23), much greater than the diameter of bovine serum albumin and nearly 1 order of magnitude greater than the diameter of ferritin. Therefore, unless the intertrabecular spaces are greatly widened in the high-voltage EM preparations, the MTL should not retard cytoplasmic diffusion strongly through solely steric effects (23). Further, we have shown here that the diffusion of macromolecules within Sepharose beads is not hindered to the extent that diffusion of the same macromolecule within the cytoplasm is. Retardation through transient binding of the diffusing species to elements of the cytoplasmic matrix is likely to be key to the explanation of the restricted diffusion rates. This conclusion also has been reached recently by Mastro *et al.* (9). The importance of this effect was recognized earlier by Paine, Horowitz, and coworkers (8, 27), who termed it the "chromatographic effect." Gershon *et al.* (23) have calculated from previous data (12) that this putative weak interaction between the diffusing species and the matrix would be characterized by a free energy of binding of about 2.5 kcal/mol.

Table 2. Modulation of DTAF-albumin cytoplasmic diffusion by chemical treatment

Treatment	D (± SEM), cm^2/sec	
	With chemical	Control
Cytolchalasin D*	$1.3\ (\pm 0.11) \times 10^{-8}$	$1.7\ (\pm 0.09) \times 10^{-8}$
Colchicine at 5°C†	$11\ (\pm 2) \times 10^{-9}$	$3.1\ (\pm 0.2) \times 10^{-9}$
Taxol‡	$1.6\ (\pm 0.13) \times 10^{-8}$	$1.7\ (\pm 0.09) \times 10^{-8}$
NaN_3/2-deoxy-glucose§	$1.9\ (\pm 0.2) \times 10^{-8}$	$1.7\ (\pm 0.2) \times 10^{-8}$

*Cytochalasin D (5 μg/ml) in P_i/NaCl for 30 min at room temperature.
†As previously reported (12). The effect of 200 μM colchicine incubated with cells in medium for 30 min at 37°C prior to FRAP determination was not experimentally significant.
‡Taxol (10 μM) was incubated with cells in medium for 22 hr (25) at 37°C prior to FRAP determination.
§NaN_3 (1 mM) and 2-deoxyglucose (50 mM) in P_i/NaCl were incubated with cells for 20 min at 37°C prior to FRAP determination.

Table 3. Comparison of cytoplasmic diffusion rates, measured by FRAP, of macromolecules in several cell types

Cell	Probe*	$D \times 10^8$, cm^2/sec	Ref.
Chicken gizzard cells	Albumin	0.6	14
	G-actin	0.3	
Human fibroblasts	Albumin	1.7	Present work
Cultured macrophages	Ovalbumin	3.5	13
Sea urchin eggs	Albumin‡	≈9.2	15
	Tubulin‡	≈7.5	
Amoebae	Albumin	≈40	13
	G-actin	10–50	

*Either fluorescein or rhodamine derivatives were used to label the probe macromolecule. Albumin, bovine serum albumin.
‡Measured by a video-photobleaching technique, but values were duplicated by spot bleaching with our instrument (unpublished data).

The view that diffusive transport of endogenous macromolecules through the cytomatrix is retarded by transient binding to elements of the matrix is made more tenable by the finding that the aqueous viscosity within the cytoplasm is only a few times that of the viscosity of water (9). Although the many surfaces of the filamentous and membraneous elements of the cytoplasm order water (28), it is likely that only the first one or two layers are rigidly bound; the remaining associated water molecules are more loosely bound, having interaction energies on the order of kT, and will retain appreciable mobility (28, 29). Hence, while this water will slow the diffusion of macromolecules to some extent, we would not expect it to totally account for the reduction in diffusion coefficients that we have measured. Indeed, the rotational diffusion of myoglobin in heart muscle was found to be reduced only to half its value in dilute solution (42). A mechanism by which diffusive transport consists of rapid aqueous jumps made between transient binding to cytomatrix elements can be thought of as diffusion through a three-dimensional lattice in which (30) $D = (1/6)\nu\lambda^2$, where λ is the mean aqueous jump length and ν is the frequency of jumping. Note that if the diffusing species is in rapid exchange with the binding elements (i.e., a "dwell" time on the cytomatrix that is short compared to the half-time for FRAP), then one can show (31) that, in order to account for the measured D, about 98% of the diffusing species will be bound at any one instant. The observed modulation of diffusion by tonicity can be qualitatively accounted for by increasing the jump distance, λ, when the cells are hypotonically swollen or by decreasing the mean distance between binding elements of the cytomatrix when the cells are shrunk hypertonically. Indeed, the MTL has been observed by high-voltage EM to swell and shrink in response to hypotonic and hypertonic treatments, respectively (32). Alternatively, the modulations of the cytoplasmic D may occur by changes in the association constant (which will affect ν) describing the interaction of the diffusing species with the cytoplasmic matrix and membranes. Changes in the salt balance during the osmotic treatments could give rise to alterations in the association constants, accounting for the effects on D.

The colchicine and cytochalasin experiments argue that depolymerization of microtubules and at least some of the F-actin is not sufficient to remove the restrictions to diffusion. These restrictions are presumably provided by remaining structures (e.g., membraneous elements, microtrabeculae, and cortical actin) whose identity remains to be elucidated.

Translational mobility within the cytoplasmic matrix. It is of interest to put the measurements made in this study in the context of the various types of translational mobility measured within the cytoplasmic matrix of tissue culture cells (see also refs. 8 and 9). The logarithm of mean distance traveled in 1 sec is plotted against the logarithm of the radius of the mobile entity in Fig. 3. For reference, the top curve indicates the behavior expected in water when diffusion is the mode of transport. Small spin labels diffuse nearly as rapidly within the matrix as they do in aqueous solution (9). A transition in mobility rates occurs (broken line) between molecular radii of 5 Å and 15 Å, which we postulate is due to the affinity of the macromolecular diffusing species for elements of the cytomatrix. As noted above, this effect has been postulated to give rise to the size-independent diffusion range (10 Å ≤ radius ≤ 100 Å). Motion of particles at least up to the 100-Å dimension appears to be thermally driven diffusion, which is not dependent on cellular energy stores. A second

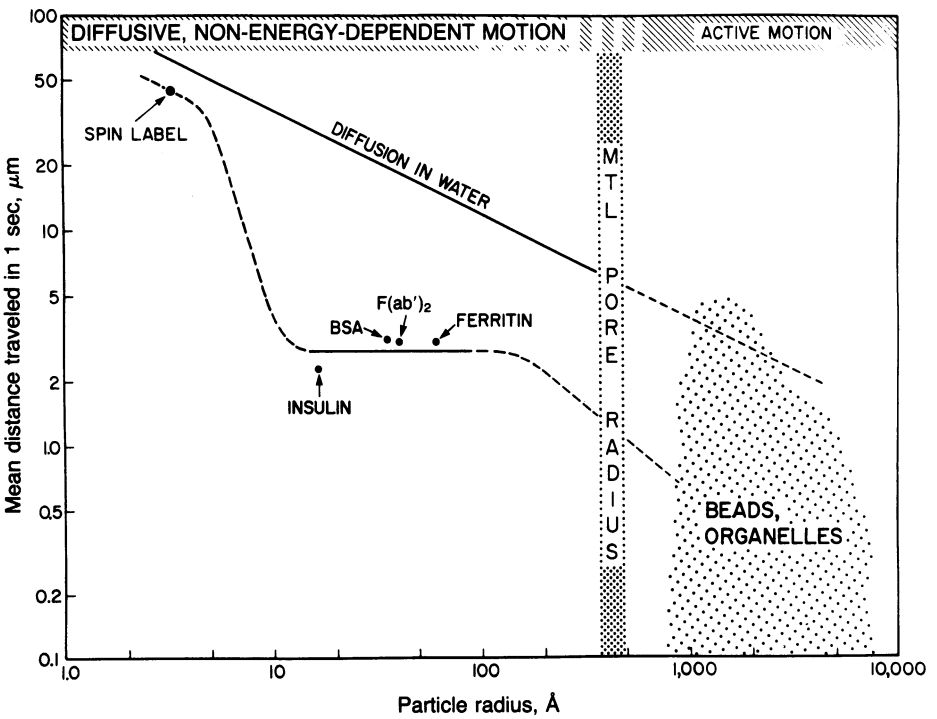

FIG. 3. Mean distance traveled in 1 sec with the cytoplasm for various sized entities. Mean pore radius of the MTL was taken from calculations of Gershon *et al.* (23). For the molecules that diffuse, mean distance traveled was taken as $(6Dt)^{1/2}$, where D was the measured diffusion coefficient and t = 1 sec. Spin-label Ds were taken from Mastro *et al.* (9), and macromolecular Ds were taken from this work. Distances diffused in water were calculated by assuming D was scaled with the radius according to the Stokes–Einstein law, given the measured values of D for bovine serum albumin (BSA) (22) and the spin label (9). In the region labeled beads and organelles, a crude estimation of the range of saltatory velocities was derived from the following works: fluorescent beads in tissue culture cells (33), plastic beads in axons (34), "cytoplasmic organelles" (35), and lysosomes (36, 37). [Note that for diagram clarity, the rapid motions (≤10 μm/sec) of unidentified submicroscopic particles (38, 39) have not been included.] Energy independence of diffusion values for bovine serum albumin and $F(ab')_2$ was tested by preincubation of BG-9 cells in 1 mM NaN_3/50 mM 2-deoxyglucose for 30 min at 37°C. Energy dependence of bead and organelle movement was inferred from the data of Adams (40) on axons and of B. Herman and colleagues (private communication) on granulosa cells.

transition may be expected as we progress to the motion of much larger particles of 1000 Å or more in radius. Such particles are larger than the putative MTL pore radius. Thus, it is reasonable that organelles and beads undergo energy-dependent, active translational movements (saltations); diffusive motions would be excluded because the particles exceed the mesh size of the matrix and, in fact, are trapped in the network.

It may be speculated that small metabolites—hormones and the like—could diffuse at nearly aqueous rates throughout the cytomatrix. Adams and Delbrück (41) estimated a significant loss in efficiency of transport from source to target caused by three-dimensional diffusion as opposed to planar or linear diffusion. Nevertheless, the diffusion of small molecules within the cytomatrix may be rapid enough to overcome this limitation; thus, bulk diffusion could be a productive means of material transport of small substances in the cytoplasm. On the other hand, our data suggest that macromolecules, in general, may undergo much slower diffusive transport at a rate that is actually determined by dissociation from cytoskeletal and membraneous binding elements. In fact, it is possible that many if not most of the cytoplasmic macromolecules are bound, albeit transiently, to the cytomatrix. It may be then that intracellular macromolecular transport is confined to the active transport of organelles in which they are enclosed or to diffusion along membranes and fibers.

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